16S Microbiome NGS Assay

Library Preparation Kit

REF 9-131 [Set A]
9-132 [Set B]
9-133 [Set C]

RUO

96 Reactions (Library Preparations)

2°C – 8°C Magnetic Beads

-30°C – -15°C Other Components

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Instructions for use

Library Preparation Kit for 16S Microbiome Analysis

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1. Introduction

The kit is intended for preparing NGS libraries for the bacterial 16S rRNA gene-based characterization of the human microbiome. In the first PCR step, the highly variable V3-V4 regions are amplified with locus-specific primers. The second PCR introduces dual index sequences for the assignment of the reads to individual samples during data demultiplexing. The final amplicon structure contains all sequences required for the analysis of the library pool on Illumina platforms (see Figure 1). This kit has been validated for the Illumina MiSeq instrument.

Graphical Workflow:

Sequencing-ready NGS libraries in < 4 hours

Variable Duration

DNA Extraction

Quantification & Dilution

ca. 1.5 hours hands-on: 10 min

PCR1 - Amplification of target region

i5 Index primer binding site

Target-specific primers

i7 Index primer binding site

16S V3-V4

Read 1/2 Primer binding sites

ca. 15 min for 12 samples

Cleanup 1

Optional: fragment size check

ca. 40 min hands-on: 10 min

PCR2 - Indexing

P7 Flow cell adapter

Index 1

16S V3-V4

Index 2

P5 Flow cell adapter

ca. 15 min for 12 samples

Cleanup 2

Optional: fragment size check

Variable Duration

Library quantification & Normalization

Pooling & Sequencing

Report generation with ViennaLab Microbiome Analysis Webtool

Included in 16S Microbiome NGS Assay

Additional reagents required

Figure 1: Graphical Workflow of the library preparation procedure

2. Kit components

[REF 9-131]

Activation Code sticker for 100 analyses [attached to the inside of the kit box lid]

Magnetic Beads	10ml
Box “16S Master Mix 2x”	5x 1ml 16S Master Mix 2x
Box “PCR1 V3-V4 Forward Primers”	8x 20µl MIB1-F1 – MIB1-F8 Primer
Box “PCR1 V3-V4 Reverse Primers”	8x 20µl MIB1-R1 – MIB1-R8 Primer
Box “PCR2 Indexing Forward Primers **Set A**”	8x 30µl MIB2-F1 – MIB2-F8 Primer
Box “PCR2 Indexing Reverse Primers Box 1/2 **Set A**”	8x 30µl MIB2-R1 – MIB2-R8 Primer
Box “PCR2 Indexing Reverse Primers Box 2/2 **Set A**”	4x 30µl MIB2-R9 – MIB2-R12 Primer

[REF 9-132]

Activation Code sticker for 100 analyses [attached to the inside of the kit box lid]

Magnetic Beads	10ml
Box “16S Master Mix 2x”	5x 1ml 16S Master Mix 2x
Box “PCR1 V3-V4 Forward Primers”	8x 20µl MIB1-F1 – MIB1-F8 Primer
Box “PCR1 V3-V4 Reverse Primers”	8x 20µl MIB1-R1 – MIB1-R8 Primer
Box “PCR2 Indexing Forward Primers **Set B**”	8x 30µl MIB2-F9 – MIB2-F16 Primer
Box “PCR2 Indexing Reverse Primers Box 1/2 **Set B**”	8x 30µl MIB2-R1 – MIB2-R8 Primer
Box “PCR2 Indexing Reverse Primers Box 2/2 **Set B**”	4x 30µl MIB2-R9 – MIB2-R12 Primer

[REF 9-133]

Activation Code sticker for 100 analyses [attached to the inside of the kit box lid]

Magnetic Beads	10ml
Box “16S Master Mix 2x”	5x 1ml 16S Master Mix 2x
Box “PCR1 V3-V4 Forward Primers”	8x 20µl MIB1-F1 – MIB1-F8 Primer
Box “PCR1 V3-V4 Reverse Primers”	8x 20µl MIB1-R1 – MIB1-R8 Primer
Box “PCR2 Indexing Forward Primers **Set C**”	8x 30µl MIB2-F17 – MIB2-F24 Primer
Box “PCR2 Indexing Reverse Primers Box 1/2 **Set C**”	8x 30µl MIB2-R1 – MIB2-R8 Primer
Box “PCR2 Indexing Reverse Primers Box 2/2 **Set C**”	4x 30µl MIB2-R9 – MIB2-R12 Primer

***Note**: REF 9-131, 9-132, 9-133 differ only in Indexing Primer Sets. If you plan to sequence more than 96 samples on one flow cell, please order different sets (e.g. Set A / REF 9-131 and Set B / REF 9-132). Make sure that all individual libraries sequenced in the same pool have a unique indexing primer combination.*

3. Equipment and reagents required but not supplied

3.1. Sample preparation

- Recommended for DNA isolation: Stool DNA Isolation Kit (Norgen Biotek).
- Other DNA isolation kits have not been validated and may lead to incorrect results.
- Recommended for DNA quantification: fluorometric methods, such as Qubit™.(Thermo Fisher)
- DNA concentration range: 1-10ng/µl.

3.2. PCR

- PCR tubes or suitable 96-well plates.
- PCR cycler with lid heating and specified ramp rates:
  heating ≤2.5°C/sec, cooling ≤1.5°C/sec.
- Nuclease-free, PCR-grade water (recommended: Illumina wash buffer PW1 or another molecular biology-grade water).

3.3. Sample purification

- 80% ethanol, freshly prepared.
- Magnetic separator for 1.5 ml microtubes, PCR strips or 96-well plates.

3.4. Library Quality Control

- Equipment and reagents to prepare 2-4% agarose gel and run the PCR products.
- Alternatively, Fragment Analyzer using DNF-473, DNF-474 or DNF-477 kit (Agilent).

3.5. Library Quantification

- Library quantification kit, e.g. JetSeq™ Library Quantification Lo-ROX Kit
  (meridian Bioscience) and associated qPCR cycler.

3.6. Sequencing

- Reagents for library denaturation and sequencing. Please order directly from Illumina.
  For MiSeq® platform, we recommend using the MiSeq Reagent kit v3 (600-cycle).
- Optional but recommended: PhiX Control v3 spiked in at 5% ratio (Illumina).

4. Laboratory protocol

4.1. First PCR – Amplification of target region

4.1.1. Reaction setup

Dilute the DNA samples to a concentration of 1-10ng/µl and set up the PCR reaction as shown in Table 1 by selecting one forward and one reverse primer for each sample. For exemplary primer combinations see Table 2.

The PCR1 forward and reverse primers contain different heterogeneity spacers ensuring sequence heterogeneity in the library pool which is necessary for Illumina sequencing. It is not essential to use a unique primer combination for each sample (e.g. the same combination can be used for samples 9-16 and samples 1-8).

Note: *Set up PCR reaction on ice.*

Table 1: Reaction Setup PCR1

Component	Volume for 1 reaction [µl]	Volume for 10 reactions incl. 5% excess [µl]
16S Master Mix 2x	25.0	262.5
V3-V4 Forward primer: MIB1-Fx	1.6	-
V3-V4 Reverse primer: MIB1-Rx	1.6	-
DNA template (1-10 ng/µl)	2.0	-
Nuclease-free water	19.8	207.9
TOTAL VOLUME	**50.0**	**470.4 (use 44.8 µl/reaction)**

Table 2: Examples for MIB1 primer combinations

Sample #	MIB1-Fx	MIB1-Rx
Sample 1	MIB1-F1	MIB1-R8
Sample 2	MIB1-F2	MIB1-R7
Sample 3	MIB1-F3	MIB1-R6
Sample 4	MIB1-F4	MIB1-R5
Sample 5	MIB1-F5	MIB1-R4
Sample 6	MIB1-F6	MIB1-R3
Sample 7	MIB1-F7	MIB1-R2
Sample 8	MIB1-F8	MIB1-R1

4.1.2. PCR program

Create a PCR program according to Table 3. Use a heated lid (>100°C); if applicable, set the heating ramp rate to max. 2.5°C and the cooling ramp rate to max. 1.5°C.

Table 3: PCR Program PCR1

Temperature	Time	Comment
95°C	03:00	Initial Denaturation
95°C	00:15	35 cycles
55°C	00:15	
72°C	00:30	
72°C	10:00	Final elongation
4°C	Hold	

4.2. Cleanup of PCR1 products

4.2.1. Preparation

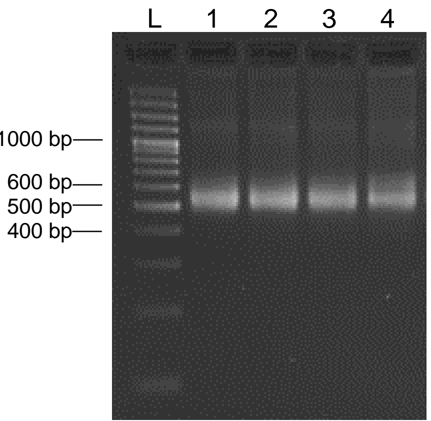
- Let the Magnetic Beads equilibrate at room temperature by transferring the bottle to the bench at least 30 min prior to use.
- Always prepare fresh 80% ethanol.
- Thoroughly resuspend the beads immediately prior to use.

4.2.2. Cleanup procedure

1. For each sample add 45µl Magnetic Beads to a tube/well.
2. Add 25µl of PCR1 product and mix well by pipetting up and down at least 10 times.
   Note: *When using less than 25µl PCR1 product, adjust the bead volume accordingly:*
   [bead] : [sample] ratio = 1.8 : 1
3. Incubate for 5 minutes at room temperature.
4. Place the tubes/plate on a magnetic stand to immobilize the beads. Incubate at room temperature until the solution is completely clear (approximately 1-2 minutes).
5. Aspirate and discard the clear supernatant without touching the beads.
6. Keep the tubes/plate on the magnetic stand and add 150µl 80% ethanol to each tube/well. It is not necessary to resuspend the beads pellet.
7. Incubate at room temperature for 30 seconds on the magnetic stand.
8. Aspirate and discard the supernatant without touching the beads.
9. Repeat Steps 6 to 8 one more time for a total of 2 washes.
10. Leave the tubes on the magnetic stand, remove residual ethanol with a pipette and dry the beads for approximately 3 minutes.
    Important: *Residual ethanol may interfere with downstream applications. Do not over-dry the beads as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte.*
11. Remove the tubes/plate from the magnetic stand.
12. Elute the samples in 25µl nuclease-free water. Mix well by pipetting up and down 10 times.
13. Incubate at room temperature for 3 minutes.
14. Place the tubes on a magnetic stand and incubate at room temperature until the beads are completely cleared from solution (approximately 1-2 minutes).
15. Transfer the cleared supernatant containing cleaned PCR products to a new tube.

4.2.3. (Optional) Check PCR1 product size (e.g. on a 2-4% agarose gel or Fragment Analyzer)

The expected size of the post-PCR1 library is approximately 550 bp (See Figure 2 and Figure 3).



L = Ladder, 1-4 = Products from PCR1

Figure 2: 4% Agarose gel of PCR1 products

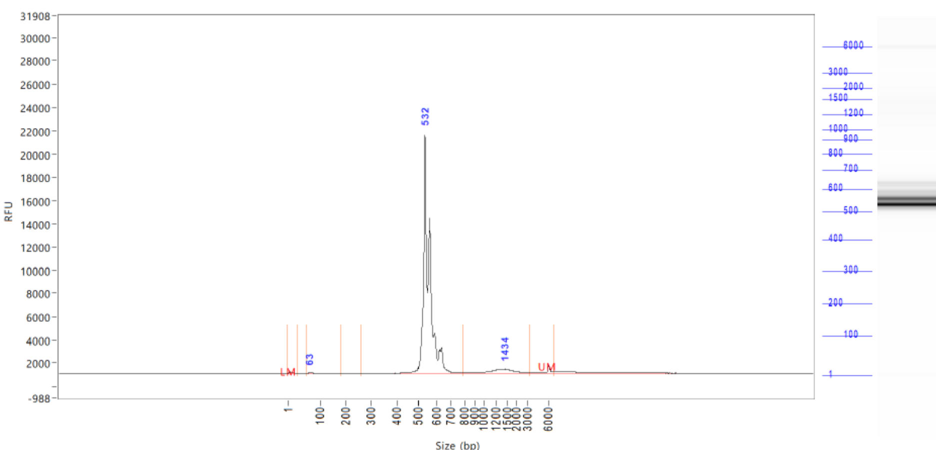


Figure 3: Fragment Analyzer result for PCR1 product

4.3. Second PCR – Indexing

4.3.1. Reaction setup

Dilute the PCR1 product 1:10 in nuclease-free water and use as template for PCR2. Set up the PCR reaction as stated in Table 4. For each sample, select a unique combination of indexing forward and reverse primers. The variation of PCR2 forward and reverse primers enables for pooling 96 samples. If a higher number of samples is intended to be pooled, please order kits with different Indexing Primer Sets.

Note: *Set up PCR reaction on ice.*
Important: *Make sure that all individual libraries sequenced in the same pool have a unique forward / reverse indexing primer combination and make note of the sample – dual-index combination (see Appendix) for the demultiplexing of the individual samples after sequencing.*

Table 4: Reaction Setup PCR2

Component	Volume for 1 reaction [µl]	Volume for 10 reactions incl. 5% excess [µl]
ViennaLab 16S Master Mix 2x	25.0	262.5
Indexing Forward primer: MIB2-Fx	1.6	-
Indexing Reverse primer: MIB2-Rx	1.6	-
Product of PCR1 (1:10 diluted)	2.0	-
Nuclease-free water	19.8	207.9
TOTAL VOLUME	**50.0**	**470.4 (use 44.8 µl/reaction)**

4.3.2. PCR program

Create a PCR program according to Table 5. Use a heated lid (>100°C); if applicable, set the heating ramp rate to max. 2.5°C and the cooling ramp rate to max. 1.5°C.

Table 5: PCR Program PCR2

Temperature	Time	Comment
95°C	03:00	Initial Denaturation
95°C	00:15	
55°C	00:15	10 cycles
72°C	00:30	
72°C	10:00	Final elongation
4°C	hold	

4.4. Cleanup of PCR2 products

4.4.1. Preparation

- Let the Magnetic Beads equilibrate at room temperature by transferring the bottle to the bench at least 30 min prior to use.
- Always prepare fresh 80% ethanol.
- Thoroughly resuspend the beads immediately prior to use.

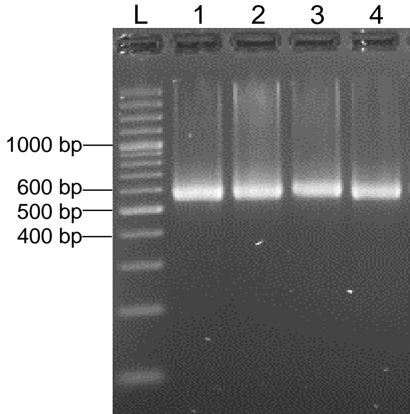
4.4.2. Cleanup procedure

1. For each sample add 45µl Magnetic Beads to a tube/well.
2. Add 25µl of PCR2 product and mix well by pipetting up and down at least 10 times.
3. **Note:** *When using less than 25µl PCR2 product, adjust the bead volume accordingly: [bead] : [sample] ratio = 1.8 : 1*
4. Incubate for 5 minutes at room temperature.
5. Place the tubes/plate on a magnetic stand to immobilize the beads. Incubate at room temperature until the solution is completely clear (approximately 1-2 minutes).
6. Aspirate and discard the clear supernatant without touching the beads.
7. Keep the tubes/plate on the magnetic stand and add 150µl 80% ethanol to each tube/well. It is not necessary to resuspend the beads pellet.
8. Incubate at room temperature for 30 seconds on the magnetic stand.
9. Aspirate and discard the supernatant without touching the beads.
10. Repeat Steps 6 to 8 one more time for a total of 2 washes.
11. Leave the tubes on the magnetic stand, remove residual ethanol with a pipette and dry the beads for approximately 3 minutes.

    Important: *Residual ethanol may interfere with downstream applications. Do not over-dry the beads as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte.*
12. Remove the tubes/plate from the magnetic stand.
13. Elute the samples in 25µl nuclease-free water. Mix well by pipetting up and down 10 times.
14. Incubate at room temperature for 3 minutes.
15. Place the tubes on a magnetic stand and incubate at room temperature until the beads are completely cleared from solution (approximately 1-2 minutes).
16. Transfer the cleared supernatant containing cleaned-up PCR products to a new tube.

4.4.3. (Optional) Check PCR2 product size (e.g. on a 2-4% agarose gel or Fragment Analyzer)

The expected size of the post-PCR2 library is approximately 600 bp (See Figure 4 and Figure 5).



L = Ladder, 1-4 = Products from PCR2

Figure 4: 4% Agarose gel of PCR2 products

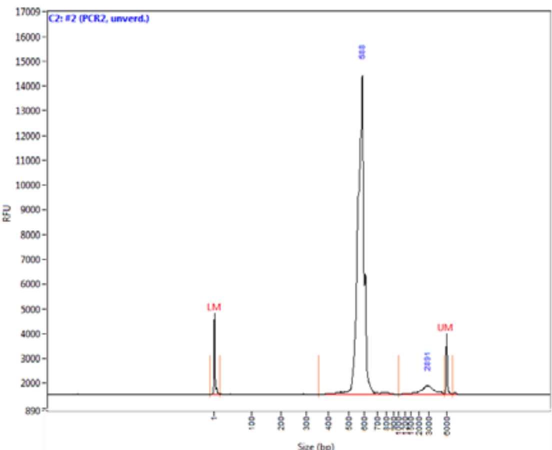


Figure 5: Fragment Analyzer result for PCR2 product

4.5. Library quantification and pooling

4.5.1. Library quantification

For the quantification of prepared libraries ViennaLab recommends the JetSeq™ Library Quantification Lo-ROX Kit (meridian Bioscience).

4.5.2. Library dilution and pooling

Dilute all individual libraries to 4 nM and pool for sequencing equimolarly or according to the required amount of data output.

For the assessment of the number of samples that can be sequenced with the used sequencing chemistry, the calculation of the sample pooling, and the assistance with the sample sheet generation, please refer to the ViennaLab MicrobeCalc™ Excel file that can be downloaded at https://www.viennalab.com/support/ngs-assays

5. Sample Sheet and sequencing recommendations

- For library pool denaturation and loading refer to Illumina System Guides.
- We recommend a spike-in of 5% PhiX.
- For sample sheet preparation see index sequences listed in Table 6 or Table 7 depending on your sequencing instrument. For MiSeq the ViennaLab MicrobeCalc™ Excel file can be used (see 4.5.2).

Table 6: Index sequences for MiSeq, MiniSeq (Rapid Reagent kits), HiSeq 2000/2500 or NovaSeq 6000 (v1.0 reagent kits)

i5 Primer	i5 index sequence	i7 Primer	i7 index sequence
MIB2-F1	ATCGTACG	MIB2-R1	CGAGAGTT
MIB2-F2	ACTATCTG	MIB2-R2	GACATAGT
MIB2-F3	TAGCGAGT	MIB2-R3	ACGCTACT
MIB2-F4	CTGCGTGT	MIB2-R4	ACTCACTG
MIB2-F5	TCATCGAG	MIB2-R5	TGAGTACG
MIB2-F6	CGTGAGTG	MIB2-R6	CTGCGTAG
MIB2-F7	GGATATCT	MIB2-R7	TAGTCTCC
MIB2-F8	GACACCGT	MIB2-R8	CGAGCGAC
MIB2-F9	CTACTATA	MIB2-R9	ACTACGAC
MIB2-F10	CGTTACTA	MIB2-R10	GTCTGCTA
MIB2-F11	AGAGTCAC	MIB2-R11	GTCTATGA
MIB2-F12	TACGAGAC	MIB2-R12	TATAGCGA
MIB2-F13	ACGTCTCG	MIB2-R13	CTCGACTT
MIB2-F14	TCGACGAG	MIB2-R14	CGAAGTAT
MIB2-F15	GATCGTGT	MIB2-R15	TAGCAGCT
MIB2-F16	GTCAGATA	MIB2-R16	TCTCTATG
MIB2-F17	TGAACCTT	MIB2-R17	GTCGTGAT
MIB2-F18	TGCTAAGT	MIB2-R18	ACCACTGT
MIB2-F19	TGTTCTCT	MIB2-R19	TGGATCTG
MIB2-F20	TAAGACAC	MIB2-R20	CCGTTTGT
MIB2-F21	CTAATCGA	MIB2-R21	TGCTGGGT
MIB2-F22	CTAGAACA	MIB2-R22	GAGGGGTT
MIB2-F23	TAAGTTCC	MIB2-R23	AGGTTGGG
MIB2-F24	TAGACCTA	MIB2-R24	GTGTGGTG

Table 7: Index sequences for iSeq100, MiniSeq (Standard reagent kits), NextSeq, HiSeq 3000/4000/X or NovaSeq 6000 (v1.5 reagent kits)

i5 Primer	i5 index sequence	i7 Primer	i7 index sequence
MIB2-F1	CGTACGAT	MIB2-R1	CGAGAGTT
MIB2-F2	CAGATAGT	MIB2-R2	GACATAGT
MIB2-F3	ACTCGCTA	MIB2-R3	ACGCTACT
MIB2-F4	ACACGCAG	MIB2-R4	ACTCACTG
MIB2-F5	CTCGATGA	MIB2-R5	TGAGTACG
MIB2-F6	CACTCACG	MIB2-R6	CTGCGTAG
MIB2-F7	AGATATCC	MIB2-R7	TAGTCTCC
MIB2-F8	ACGGTGTC	MIB2-R8	CGAGCGAC
MIB2-F9	TATAGTAG	MIB2-R9	ACTACGAC
MIB2-F10	TAGTAACG	MIB2-R10	GTCTGCTA
MIB2-F11	GTGACTCT	MIB2-R11	GTCTATGA
MIB2-F12	GTCTCGTA	MIB2-R12	TATAGCGA
MIB2-F13	CGAGACGT	MIB2-R13	CTCGACTT
MIB2-F14	CTCGTCGA	MIB2-R14	CGAAGTAT
MIB2-F15	ACACGATC	MIB2-R15	TAGCAGCT
MIB2-F16	TATCTGAC	MIB2-R16	TCTCTATG
MIB2-F17	AAGGTTCA	MIB2-R17	GTCGTGAT
MIB2-F18	ACTTAGCA	MIB2-R18	ACCACTGT
MIB2-F19	AGAGAACA	MIB2-R19	TGGATCTG
MIB2-F20	GTGTCTTA	MIB2-R20	CCGTTTGT
MIB2-F21	TCGATTAG	MIB2-R21	TGCTGGGT
MIB2-F22	TGTTCTAG	MIB2-R22	GAGGGGTT
MIB2-F23	GGAACTTA	MIB2-R23	AGGTTGGG
MIB2-F24	TAGGTCTA	MIB2-R24	GTGTGGTG

6. Quick Guide to sequencing data analysis

6.1. **a)** Upon first use please navigate to https://microbiome.viennalab.com and register for the ViennaLab Microbiome Analysis Webtool providing a valid email address and the Activation Code you find on the inner side of the kit box lid.
b) If you are an already registered user, please enter the Activation Code provided with the kit in your Account profile.

6.2. Upload the raw sequencing data files. Supported file formats include *.fastq and *.fastq.gz. If you have paired input files, check the box "Paired input files" and click on Upload.

6.3. You will then be asked to confirm the deduction of 1 sample credit from your balance for each single or two paired sequencing data files by clicking on Upload and Analyze.

***Important**: Do not close the browser window or redirect or reload the page while files are being uploaded, as this would interrupt the upload procedure.*

6.4. When upload and processing are finished sample status will turn from "Processing" to "Ready".

6.5. You can now review your results and generate reports.

For technical support please contact ViennaLab through the local distributor or directly at techhelp@viennalab.com.

APPENDIX

Tables for used indexing primer combinations: check which index combinations have already been used or enter sample name for later identification.

[9-131]

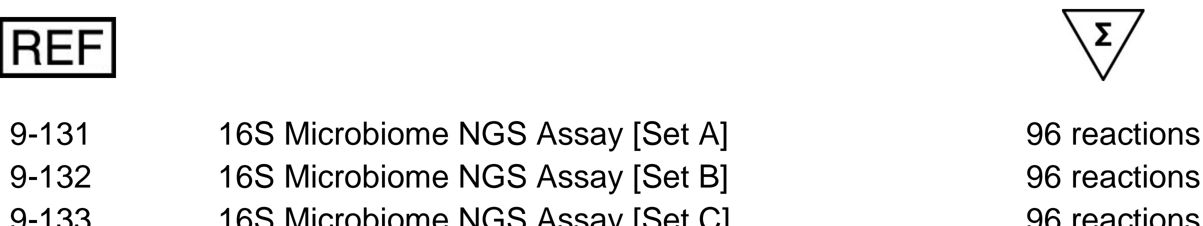
MIB2-	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
F1												
F2												
F3												
F4												
F5												
F6												
F7												
F8												

[9-132]

MIB2-	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
F9												
F10												
F11												
F12												
F13												
F14												
F15												
F16												

[9-133]

MIB2-	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
F17												
F18												
F19												
F20												
F21												
F22												
F23												
F24												



REF		Σ
9-131	16S Microbiome NGS Assay [Set A]	96 reactions
9-132	16S Microbiome NGS Assay [Set B]	96 reactions
9-133	16S Microbiome NGS Assay [Set C]	96 reactions

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